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Minimal influence of G-protein null mutations on ozone-induced changes in gene expression, foliar injury, gas exchange and peroxidase activity in *Arabidopsis thaliana* L

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Abstract

Ozone (O_3) uptake by plants leads to an increase in reactive oxygen species (ROS) in the intercellular space of leaves and induces signalling processes reported to involve the membrane-bound heterotrimeric G-protein complex. Therefore, potential G-protein-mediated response mechanisms to O_3 were compared between *Arabidopsis thaliana* L. lines with null mutations in the α- and β-subunits (*gpa1-4, agb1-2* and *gpa1-4/agb1-2*) and Col-0 wild-type plants. Plants were treated with a range of O_3 concentrations (5, 125, 175 and 300 nL L^{-1}) for 1 and 2 d in controlled environment chambers. Transcript levels of *GPA1*, *AGB1* and *RGS1* transiently increased in Col-0

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effects of (a) assay buffer and (b) pH on DAF peroxidase activity in extracts of leaf tissue from wild-type Col-0 *Arabidopsis* plants. (a) DAF peroxidase activity in different assay buffers at pH 5.0. (b) DAF peroxidase activity in potassium phosphate (KPi) and sodium acetate (NaOAc) buffer solutions at a range of pH values. Values are LSMEANS ± SE. The reaction rate of the assay was stable at temperatures between 25 to 35 °C (data not shown).

Table S1. Photosynthesis, oxidative stress, ROS-induced and PCD-signalling genes up- or down-regulated at 3 h and 2 d after exposure of Col-0 and *gpa1-4/agb1-2* plants to 125 nL L^{-1} O_3 (two-way ANOVA, $P \leq 0.05$) (ns, not statistically significant). O_3 × genotype interactions were not statistically significant ($P \geq 0.05$). Fold-change values are averages of Col-0 and *gpa1-4/agb1-2* responses.

Table S2. Phytohormone biosynthesis and related response genes up- or down-regulated at 3 h and 2 d after exposure of Col-0 and *gpa1-4/agb1-2* plants to 125 nL L^{-1} O_3 (two-way ANOVA, $P \leq 0.05$) (ns, not statistically significant). O_3 × genotype interactions were not statistically significant ($P \geq 0.05$). Fold-change values are averages of Col-0 and *gpa1-4/agb1-2* responses.

Table S3. Peroxidase activities (nkats mg $protein^{-1}$) in leaf tissue extracts of Col-0 plants treated for 2 d with 5, 125 or 175 nL L^{-1} O_3 7 h daily. Activities were measured using three different substrates and H_2O_2. Values are the LSMEANS ± SE. Significant differences between elevated O_3 and the control treatment (5 nL L^{-1}), within an assay substrate, are indicated as: **, $P \leq 0.01$; *, $P \leq 0.05$.

Table S4. Peroxidase kinetic parameters (K_m and V_{max}) for Col-0 and *gpa1-4/agb1-2* genotypes in extracts of leaf tissues from plants treated for 2 d with either 5 or 125 nL L^{-1} O_3 7 h d^{-1}. Affinity for both peroxidase substrates [diaminofluorene (DAF) and H_2O_2] was calculated using Hanes and Woolf's plots ([substrate]/rate versus [substrate]), according to the Michaelis-Menten kinetics equation. Values are the LSMEANS ± SE. Significant differences between O_3 treatments within a genotype are indicated as: *, $P \leq 0.05$.

Table S5. Gene expression changes at 3 h and 2 d after exposure of Col-0 and *gpa1-4/agb1-2* plants to 125 ppbv ozone ($P \leq 0.05$) (ns, not significant). Ozone × genotype interactions were not statistically significant ($P \geq 0.05$). Fold-change values are averages of Col-0 and *gpa1-4/agb1-2* responses.

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exposed to 125 nL L^{-1} O_3 compared with the 5 nL L^{-1} control treatment. However, silencing of α and β G-protein genes resulted in little alteration of many processes associated with O_3 injury, including the induction of ROS-signalling genes, increased leaf tissue ion leakage, decreased net photosynthesis and stomatal conductance, and increased peroxidase activity, especially in the leaf apoplast. These results indicated that many responses to O_3 stress at physiological levels were not detectably influenced by α and β G-proteins.

Keywords

conductance; G-proteins; oxidative stress; ozone, peroxidase; photosynthesis; ROS; stomata

INTRODUCTION

Ambient ozone (O_3) concentrations in many regions worldwide can suppress crop productivity, damage forests and impair ecosystem health (Fuhrer, Skarby & Ashmore 1997; Booker *et al.* 2009). Global tropospheric O_3 concentrations range from 25 to 50 nL L^{-1}, with higher daily concentrations occurring regionally (Fowler *et al.* 1999). In the United States, the seasonal 8 h average O_3 concentration is 45 to 50 nL L^{-1} (http://www.epa.gov/airtrends/weather.html). Concentrations in the range of 40 nL L^{-1} and higher can cause visible injury and growth suppression in sensitive plants (Booker *et al.* 2009; Mills *et al.* 2011). While much is known about phenotypic plant responses to chronic O_3 exposure, less is understood about the biochemical and molecular basis of these effects, especially at concentrations typical of O_3-polluted regions of the world (Fiscus, Booker & Burkey 2005; Heath 2008).

Upon entering the leaf intercellular space through the stomata, O_3 dissolves in the intercellular solution surrounding the cells and reacts with cellular components, which can cause injury and produce additional reactive oxygen species (ROS) (e.g. OH., $^1O_2$, $O_2^{\cdot-}$ and H_2O_2) (Mudd 1996; Moldau 1998). Membrane proteins with easily oxidized residues (Cys, Met, Try and Trp) at active sites are preferentially susceptible to O_3 (Mudd 1996). However, cell damage is also under genetic control, rather than only a result of ROS toxicity (op den Camp *et al.* 2003; Kangasjarvi, Jaspers & Kollist 2005; Mullineaux & Baker 2010). Genetic control of cell death includes pro- and anti-cell death signalling, possibly involving G-proteins (Rao & Davis 2001; Mullineaux & Baker 2010).

In plants, the membrane-bound heterotrimeric G-protein signal pathway participates in a host of morphological, metabolic, physiological and gene expression responses that can affect gas-exchange, redox status, ion regulation and hormone interactions (Joo *et al.* 2005; Van Breusegem, Bailey-Serres & Mittler 2008;Wang, Assmann & Fedoroff 2008; Zhang *et al.* 2008; Okamoto *et al.* 2009; Trusov *et al.* 2009; Nilson & Assmann 2010; Pandey *et al.* 2010; Zhang, Jeon & Assmann 2011). Using loss-of-function *Arabidopsis* mutants in the G-protein signalling pathway, it was found that the bimodal H_2O_2 burst following an acute O_3 exposure in the ecotype Col-0 was partly regulated by G-proteins (Joo *et al.* 2005). Specifically, the α- and β-subunit null mutants exhibited a suppressed initial burst while in the α-subunit null mutant, the subsequent burst was also attenuated (Joo *et al.* 2005). This suggests that changes in signalling processes from O_3 might be influenced by G-proteins. G-

protein signalling can also be initiated by H_2O_2, which causes the α-subunit to dissociate from the heterotrimeric complex (Wang *et al*. 2008).

Plant peroxidases reduce H_2O_2 and other hydroperoxides produced by exposure to O_3 (Langebartels *et al*. 2002; Passardi *et al*. 2005). Peroxidases have a prominent role in studies of plant responses to O_3, partly because they are relatively easy to measure and because activity changes are usually large and consistently found. For more than 40 years, peroxidase activity has been reported to increase in response to both chronic and acute O_3 exposures in many plant species (Curtis, Howell & Kremer 1976; Castillo & Greppin 1986; Kubo *et al*. 1995; Burkey *et al*. 2000; Ranieri *et al*. 2000b; Cheng *et al*. 2007). Native polyacrylamide gel electrophoresis (PAGE) often showed increased activity of various peroxidase isozymes in response to acute O_3 exposures (Curtis *et al*. 1976; Ranieri, Castagna & Soldatini 2000a; Ranieri *et al*. 2000b; Scebba *et al*. 2003). In addition, increases in extracellular peroxidase activity following acute O_3 exposure were measured in *Sedum album* L., pinto bean (*Phaseolus vulgaris* L.) and sunflower (*Helianthus annuus* L.) leaves (Castillo & Greppin 1986; Peters, Castillo & Heath 1989; Ranieri *et al*. 2000b). Northern blotting and gene expression array analyses with *Arabidopsis* showed that transcripts for ascorbate peroxidase (APX) and guaiacol peroxidases were increased by O_3 (Sharma & Davis 1994; Conklin & Last 1995; Kubo *et al*. 1995; Tamaoki *et al*. 2003; Mahalingam *et al*. 2005; Ludwikow & Sadowski 2008). Changes in gene expression and activities of enzymes that modulate peroxide levels, such as peroxidases, might thus be influenced via signalling processes that involve G-proteins. Alternatively, O_3 effects on H_2O_2 concentrations might affect G-protein signalling (Wang *et al*. 2008).

Given that the plant G-protein complex was reported to mediate O_3 responsiveness, the objective of this study was to determine how G-proteins influence plant responses to O_3 in *Arabidopsis*. Effects on G-protein gene expression, as well as effects of α- and β-subunit null mutations on gene expression, physiological and biochemical responses to a range of O_3 concentrations, were examined. The genotypes tested were wild-type (Col-0) and G-protein null mutants *gpa1-4, agb1-2* and the *gpa1-4/agb1-2* double mutant. Measured responses included transcriptome changes, leaf tissue ion leakage, net photosynthesis, stomatal conductance and density, and total leaf and extracellular peroxidase activities.

METHODS

Genotypes, plant culture, O_3 treatments and plant sampling

Genotypes used in this study were Col-0 and G-protein null mutants *gpa1-4* (Jones, Ecker & Chen 2003), *agb1-2* and *gpa1-4*/*agb1-2* (Ullah *et al*. 2003). All tDNA insertion mutants were in the Col-0 background.

Seeds were sown in small pots containing Metro-Mix (Sun Gro Horticulture, Bellevue, WA, USA), stratified for 3 d at 4 °C and then germinated under a photosynthetic photon flux density (PPFD) of 400 μmol m^{-2} s^{-1} (9 h light/15 h dark cycle) at 23 °C in a growth chamber in the North Carolina State University Phytotron. Seedlings were transplanted into Metro-Mix in 5 × 5 × 5 cm cell-packs and grown for 4–5 weeks in the growth chamber.

Plants were fertilized with Phytotron nutrient solution (Downs & Thomas 1983) once per week.

On the day preceding an O_3 exposure, plants were transferred to four continuous-stirred tank reactor (CSTR) chambers located in a walk-in growth chamber in the North Carolina State University Phytotron (Booker *et al.* 2004). The CSTRs are Teflon-covered cylindrical chambers designed for the rapid mixing of gases. Plants were watered and allowed to acclimate overnight (~16 h). Temperature, relative humidity (RH) and PPFD in the chambers were 24 °C, 53% and 362 μmol m^{-2} s^{-1}, respectively. Ozone treatments consisted of charcoal-filtered air mixed with the desired amount of O_3 generated by electrical discharge in dry O_2 (model TG-10, Ozone Solutions, Inc., Hull, IA, USA). Average O_3 concentrations were 5, 125, 175 or 300 nL L^{-1} O_3 (7 h d^{-1}). Ozone concentrations were measured every 8 min using a UV O_3 monitor (Model 49C, Thermo Scientific, Franklin, MA, USA). Ozone was administered by a computer-controlled feedback monitoring and dispensing system utilizing mass-flow control valves.

Following O_3 treatment 7 h d^{-1} for up to 2 d, fully expanded, mature leaves (mid-whorl) were sampled for analysis. Leaf tissues used for gene expression and peroxidase assays were frozen in liquid N_2 and kept at −80 °C until assayed.

Gene expression array

Total RNA in leaf tissue samples (50 mg) obtained after exposure to 5 or 125 nL L^{-1} O_3 for 3 h and 2 d was extracted using Qiagen RNeasy mini kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA was quantified by measuring A_{260} on a spectrophotometer and integrity was checked using RNA Nano LabChips and Model 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Labelled cRNAs were synthesized at the Functional Genomics Core Facility, University of North Carolina – Chapel Hill and hybridized to the GeneChip Arabidopsis ATH1 Genome Array (Affymetrix, Santa Clara, CA, USA). Total RNA (1 μg) was used to synthesize cDNA followed by aRNA. The MessageAmp II-Biotin Enhanced Kit (Ambion, Invitrogen Corp., Carlsbad, CA, USA) was used to generate biotinylated aRNA from the cDNA reaction. The aRNA was then fragmented in fragmentation buffer from the Ambion kit at 94 °C for 35 min. Fragmented aRNA (15 μg) was then added to a hybridization cocktail (0.05 μg μL^{-1} fragmented cRNA; 50 pM control oligonucleotide B2; *BioB*, *BioC*, *BioD* and *cre* hybridization controls; 0.1 mg mL^{-1} herring sperm DNA; 0.5 mg mL^{-1} acetylated BSA; 100 mM MES; 1 M [Na+], 20 mM EDTA; and 0.01% Tween 20). aRNA (10 μg) was used for hybridization in a volume of 200 μL per slide. Affymetrix arrays were hybridized for 16 h at 45 °C in a GeneChip Hybridization Oven 640 (Affymetrix). The arrays were washed and stained with R-phycoerythrin streptavidin in the GeneChip Fluidics Station 450 (Affymetrix) using wash protocol EukGE-WS2v4, and arrays were scanned with the GeneChip Scanner 3000 7G Plus with autoloader. Affymetrix Gene-Chip Operating Software was used for washing, scanning and basic analysis. Sample quality was assessed by examination of 3′ to 5′ intensity ratios of certain genes.

Gene expression results were analysed using Genespring GX (Ver. 11.5.1, Agilent Technologies). Probe-level intensity measurements were summarized by Robust Multi-array

Analysis (Irizarry *et al.* 2003), and a log base 2 baseline transformation to the median of all samples was performed for each sampling time. Data were statistically analysed using an unpaired *t*-test (genotype) and a two-way (O_3 × genotype) ANOVA with 1.45-fold-change cutoff values (Tosti *et al.* 2006). The Benjamini Hochberg FDR procedure at a cutoff value of $P \leq 0.05$ was used for multiple testing correction. There were three and four independent biological replicate samples (individual plants from separate chambers and experiments) for each treatment combination at the 3 h and 2 d sampling times, respectively.

Ion leakage experiment

Immediately following an O_3 treatment for 1 d, leaves (0.2 g fresh weight) were cut at the petiole and placed in 5 mL of H_2O in a 15 mL polypropylene tube. The samples were allowed to incubate at room temperature for 2.5 h. Afterward, leaf samples were transferred to 5 mL of H_2O in a second tube, sealed and autoclaved. Conductivity of the incubation and autoclaved solutions was measured with a conductivity meter (model 4403, Markson Science, Inc., Del Mar, CA, USA). Relative ion leakage was expressed as incubation solution conductivity / (incubation solution + autoclaved solution conductivities).

Photosynthesis, stomatal conductance and stomatal density

Net photosynthesis (A_n) and stomatal conductance (g_s) of mid-whorl leaves were measured using a portable photosynthesis system fitted with a sampling chamber with a 0.79 cm^2 aperture (model Li-6400 and Li-6400-15, Li-Cor, Inc., Lincoln, NE, USA). The leaf cuvette was illuminated by overhead lights in the growth chamber. Air flow to the cuvette was set at 300 μmol s^{-1}. During the measurements, [CO_2], leaf temperature, VPD_{leaf}, RH and PPFD in the leaf chamber averaged (± SD) 370 ± 1 μmol mol^{-1}, 24.8 ± 0.5 °C, 1.66 ± 0.13 kPa, 42 ± 2% and 524 ± 33 μmol m^{-2} s^{-1}, respectively. Measurements were made after 1 and 2 d of O_3 treatments.

Stomatal density was determined by examination of the abaxial epidermis from mid-whorl leaves under bright field microscopy (model BH-2, Olympus America, Inc., Center Valley, PA, USA). Epidermes were obtained by immobilizing 0.5 × 1.0 cm sections of leaf tissue, abaxial side down, on adhesive cellophane tape and then gently scraping away the adaxial epidermis and mesophyll tissue with a razor blade, leaving the abaxial epidermis adhered to the tape. The tape was then applied to a microscope slide with the abaxial surface of the epidermis facing upward. Stomatal counts were made on digitized images showing 0.36 mm^2 of leaf area at 360× magnification. Images were acquired with an Olympus Q-Color3 camera and Qcapture 2.7 software (Qimaging Corporation, Surrey, BC, Canada). Tissue samples were obtained from two leaves of each of five plants per genotype. Image quality and resolution was comparable to images obtained with epidermal peels or leaf impressions (Gitz & Baker 2009).

Intercellular washing fluid extraction

Intercellular washing fluid (IWF) extraction was conducted as described by Burkey (1999), with the following modifications. Leaves (0.7–1.0 g fresh weight) were placed in a 60 mL polypropylene syringe and submerged in 25 mL of 100 mM potassium phosphate buffer (KPi) (pH 6.5). Extraction buffer used for APX assays also contained 10 mM ascorbic acid

(AA) to stabilize enzyme activity. Leaves were infiltrated under vacuum followed by compression using the syringe plunger. The leaves were blotted dry, weighed and placed in a 5 mL syringe barrel nested inside a 15 mL polypropylene centrifuge tube. The tip of the 5 mL syringe barrel was fitted into a 1.5 mL screw-top microcentrifuge tube placed in the bottom of the 15 mL tube. The tubes were spun at 550 *g* in a swinging-bucket rotor in a table-top centrifuge for 4 min. Afterward, IWF samples collected in the 1.5 mL microcentrifuge tubes were mixed and frozen at −20 °C for later analysis. Possible contamination of IWF with intracellular components was evaluated by measuring for the presence of glucose-6-phosphate in IWF samples, as previously described (Burkey 1999). Extraction yield of infiltrated leaf tissues averaged 97.5 ± 0.2% based on fresh weights of leaf tissue samples before and after centrifugation.

Peroxidase assays

Leaf tissue samples (100 mg fresh weight) were homogenized in 1.5 mL of ice-cold 50 mM KPi buffer solution (pH 6.5) in a chilled mortar containing 10 mg of 50% polyvinylpolypyrrolidone/50% acid-washed sand (v/v). For APX assays, the KPi buffer solution contained 10 mM AA. Samples were then centrifuged for 8 min at 20 000 *g* and the supernatant was collected. Peroxidase activity measurements of IWF samples were obtained following centrifugation of thawed samples at 4 °C.

Peroxidase activity was measured using the following substrates, as described previously, with modifications: guaiacol (Cheng *et al.* 2007), syringaldazine (Castillo & Greppin 1986) and 2,7-diaminofluorene (DAF) (Criquet, Joner & Leyval 2001). Peroxidase assays with guaiacol as a substrate were conducted in 50 mM KPi buffer (pH 6.5) containing 10 mM H_2O_2, 40 mM guaiacol and 25 μL of plant extract. Activity was determined by measuring the increase in absorbance at 436 nm at 25 °C for 30 s, using $\varepsilon = 25.5\ mM^{-1}\ cm^{-1}$. Peroxidase assays with syringaldazine were conducted in 50 mM KPi buffer (pH 6.5) containing 5 mM H_2O_2 and 50 μM syringaldazine dissolved in dimethylsulfoxide (DMSO). Activity was determined by measuring the increase in absorbance at 530 nm at 25 °C for 30 s, using $\varepsilon = 65\ mM^{-1}\ cm^{-1}$ (Harkin & Obst 1973). For DAF measurements, enzyme activity was optimized (Supporting Information Fig. S1) to develop a routine assay consisting of a 25 μL aliquot of plant extract added to 50 mM sodium acetate (NaOAc) buffer (pH 5.0) containing 5 mM H_2O_2 and 1.25 mM DAF dissolved in DMSO. Absorbance change at 600 nm was measured at 25 °C for 30 s in a spectrophotometer following a 10 s delay. An experimentally determined extinction coefficient for oxidized DAF (fluorine blue, $\varepsilon_{600} = 9.84\ mM^{-1}\ cm^{-1}$) was used to calculate enzyme activity. Ozone-induced peroxidase activity was evident with all three substrates (Supporting Information Table S3) with the highest rates observed for DAF, showing that DAF was a suitable peroxidase substrate with potentially greater sensitivity than guaiacol and syringaldazine, and without the precipitation problem that can occur with syringaldazine.

For APX assays, a 50 μL aliquot of plant extract was added to 100 mM Hepes-KOH buffer (pH 7.0) containing 0.4 mM H_2O_2, 0.2 mM AA and 0.1 mM EDTA. Absorbance change at 290 nm was measured at 25 °C for 30 s in a spectrophotometer following a 15 s delay. Enzyme activity was directly proportional to absorbance measured as $\varepsilon_{290} = 2.8\ mM^{-1}\ cm^{-1}$.

Total protein concentration of each plant extract sample was measured by the modified Bio-Rad method (Bradford 1976). All enzyme and protein assays were conducted in duplicate.

Enzyme kinetics were measured for both DAF and H_2O_2 while the other substrate was held at saturating conditions. Leaf tissue extracts were desalted with 50 mM NaOAc (pH 5.0) buffer using Sephadex PD-10 columns according to manufacturer recommendations (Pharmacia Biotech, Uppsala, Sweden). Aliquots of column eluent were assayed for DAF activity as described previously. The maximum reaction rate (V_{max}) and affinity coefficient (K_m) were calculated using Hanes-Woolf analysis (Segel 1975).

Experimental design and statistics

Results were analysed as a split-plot with O_3 treatments as the main plot and genotype as the subplot using a mixed model analysis (Littell *et al.* 1996) (PC SAS for Windows, ver. 9.2). To meet normality and homogeneity of variance constraints, some data (g_s, enzyme activities) were natural log transformed. Reported values are the LSMEANS and associated standard error of the LSMEANS. Statistically significant differences are from a *priori* pairwise, linear contrasts.

RESULTS

Gene expression analysis

Overall, 4015 statistically significant changes in gene expression were found following exposure to 125 nL L^{-1} O_3 for 3 h and/or 2 d ($P \leq 0.05$) (Supporting Information Table S5). Few differences in gene expression were observed between Col-0 and *gpa1-4/agb1-2* genotypes (Table 1). As expected, expression of *GPA1* and *AGB1* in the *gpa1-4/agb1-2* null mutant was much lower than in Col-0 plants. Stress-response genes related to cold- or water-stress tolerance (*COR47*) and far-red light perception (*FAR1*) were up-regulated in the null mutantcompared with Col-0 after O_3 exposure for 3 h. After 2 d of O_3 exposure, expression of a PR-6 type protein encoding gene was down-regulated 2.9-fold by O_3 in the null mutant compared with Col-0. In contrast, expression of phosphoglycerate/bisphosphoglycerate mutase (At3g05170) was up-regulated 3.0-fold in the null mutant after 2 d compared with Col-0. This enzyme has a central role in glycolysis. Differential expression of three unknown genes was also observed between the null mutant and Col-0 in response to O_3.

Transcription of G-protein subunits *GPA1* and *AGB1* was up-regulated in Col-0 after exposure to 125 nL L^{-1} O_3 for 3 h, but not after a 2 d exposure (Table 2). Expression of *REGULATOR OF G-PROTEIN SIGNALING 1* (*RGS1*) was also increased by O_3 after 3 h in Col-0 and the double mutant, but not after 2 d.

Despite evidence that the steady-state level of G-protein transcripts increased, other transcriptome changes often associated with O_3 exposure all apparently occurred independently of α- and β-subunit-dependent G-protein signalling processes. Gene expression changes in photosynthesis, ROS signalling, oxidative stress responses and phytohormone biosynthesis due to O_3 (Supporting Information Tables S1 & S2) were not significantly different between Col-0 and *gpa1-4/agb1-2* genotypes ($P \geq 0.05$).

We found that APX1 gene expression remained unchanged with O_3 exposure in contrast to a number of reports in the literature to the contrary (Conklin & Last 1995; Kubo *et al*. 1995; Ludwikow, Gallois & Sadowski 2004). Several other APX genes were down-regulated by O_3 (Table 3). Expression of dehydroascorbate reductase, which participates in AA regeneration, was down-regulated after 3 h, but up-regulated after 2 d. Responses of various other peroxidases following 3 h O_3 exposure were mixed, but generally they were up-regulated by O_3 after 2 d (Table 3). Expression of several peroxiredoxins and thioredoxins was lowered by O_3, although glutaredoxin was up-regulated (Supporting Information Table S1). Numerous glutathione-S transferase genes were strongly up-regulated by O_3 as well. NAD(P)H oxidase transcription was up-regulated by O_3, as was ascorbate oxidase (Supporting Information Table S1).

Several genes that respond to ROS production were up-regulated by O_3 (Supporting Information Table S1). These included *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*) and *ARABIDOPSIS THALIANA NUDIX HYDROLASE7* (*ATNUDT7*), which play mutually antagonistic roles in the control of antioxidant and other defences against cell death, including regulation of salicylic acid (SA) biosynthesis (Mullineaux & Baker 2010). Up-regulation of *FLAVINDEPENDENT MONOOXYGENASE* (*FMO1*), which encodes a detoxification enzyme and is a marker for programmed cell death (PCD) (Olszak *et al*. 2006), was also observed after O_3 treatment for 2 d. Increased expression of *BON ASSOCIATION PROTEIN1* (*BAP1*), a marker for $^1O_2$ and negative regulator of PCD (Ochsenbein *et al*. 2006;Yang *et al*. 2007), was detected after O_3 treatment for 2 d, but interestingly, expression of *FERRITIN1* (*FER1*), a marker for H_2O_2 (Ochsenbein *et al*. 2006), was not significantly altered by O_3. *METACASPASE1* (*MC1*), a positive regulator of PCD, was up-regulated by O_3 after 3 h, while expression of *METACASPASE2* (At4g25110, *MC2*), a negative regulator of PCD, was unchanged. A number of mitogen-activated protein kinase (MAPK) and MAP kinase kinase (MAPKK) genes were transcriptionally up-regulated by O_3 after 3 h, including the previously reported *AtMPK3, AtMPK6* and *AtMPK11* (Kangasjarvi *et al*. 2005; Tosti *et al*. 2006) (Supporting Information Table S1). Transcription of *AtMPK11* was strongly enhanced. Ozone exposure up-regulated *AtMKK4*, *AtMKK5* and *AtMKK9*, which have been shown to activate *AtMPK3* and *AtMPK6*, and participate in disease-resistance pathways and the hypersensitive response (Hua, Yang & Fromm 2006).

Increased expression of genes involved in the biosynthesis of ethylene (ET), jasmonic acid (JA) and SA was observed in response to O_3 (Supporting Information Table S2). Several ET-, JA- and SA-mediated signalling genes were up-regulated as well as two pathogenesis-related genes (*PR1* and *PR5*). *ARABIDOPSIS NONEXPRESSOR OF PR GENES1* (*NPR1*), which is required for SA-induced *PR* gene expression (Dong 2004), was up-regulated after O_3 exposure for 3 h, followed by massive up-regulation of *PR* genes by day 2. Decreased expression of a polyamine oxidase gene, which encodes an enzyme that catabolizes polyamines, also occurred in response to O_3.

Ion leakage assay

As shown in Fig. 1, treatment with 175 and 300 nL L^{-1} O_3 for 7 h increased ion leakage from leaves in all genotypes tested ($P \leq 0.001$), with some small differences among genotypes. Relative ion leakage from *agb1-2* samples in the 175 nL L^{-1} O_3 treatments was 24% higher than in Col-0 while leakage from *agb1-2* and *gpa1-4/agb1-2* was slightly higher in the 300 nL L^{-1} treatment compared with Col-0 (O_3 × genotype interaction, $P \leq 0.05$). Relative ion leakage in the 125 nL L^{-1} O_3 treatment was not significantly different from controls (5 nL L^{-1} treatment), and there were no significant differences in ion leakage among genotypes in the 5 nL L^{-1} treatment.

No visible injury was evident in plants treated with either 5 or 125 nL L^{-1} O_3. Minor injury was observed occasionally on plants treated with 175 nL L^{-1}, while leaves from the 300 nL L^{-1} treatment often exhibited partial tissue collapse, followed the next day by necrosis. No difference in the nature or extent of visible injury was discernable among the various genotypes.

Net photosynthesis, stomatal conductance, O_3 flux and stomatal density

After 1 or 2 d of treatment with 125 or 175 nL L^{-1} O_3, net photosynthesis (A_n) was suppressed by 29% among all genotypes ($P \leq 0.001$) (Fig. 2a). Differences in A_n between the elevated O_3 concentrations were not statistically significant ($P \geq 0.05$). Net photosynthesis was 12% lower on the second day of treatment compared with the first day ($P \leq 0.05$) (data not shown). Net photosynthesis rates were significantly different among genotypes ($P \leq 0.05$), but the range of A_n was ≤ 8% when averaged across O_3 treatments.

Stomatal conductance (g_s) was suppressed by 46% on average in the 125 and 175 nL L^{-1} O_3 treatments after 1 or 2 d ($P \leq 0.001$) (Fig. 2b). Relative g_s was 20% lower on the second day of treatment compared with the first day ($P \leq 0.05$) (data not shown). There were no statistically significant differences in g_s among genotypes or between the elevated O_3 treatments ($P \geq 0.05$).

Intercellular CO_2 concentrations (C_i) in the control (5 nL L^{-1} O_3) treatment were 282 ± 17 and 304 ± 17 μmol mol^{-1} after 1 and 2 d of the experiment, respectively ($P \leq 0.001$). Intercellular CO_2 concentrations in the elevated O_3 treatments were 6 to 8% lower compared with the control ($P \leq 0.001$) (data not shown). There were no statistically significant differences in C_i among genotypes or between the elevated O_3 treatments ($P \geq 0.05$).

Stomatal density was 43% lower in *gpa1-4* plants compared with Col-0 (Table 4). Stomatal density in *agb1-2* was 27% higher than Col-0, while *gpa1-4/agb1-2* densities were similar to Col-0.

Leaf tissue peroxidase responses to O_3

APX activities in leaf tissue extracts increased by 32 to 47% in all genotypes except *agb1-2* following treatment with 175 nL L^{-1} O_3 for 1 or 2 d ($P \leq 0.05$) (Fig. 3a,b). Effects of the 125 nL L^{-1} treatment were not statistically significant.

After exposure to 175 nL L^{-1} O_3 for 1 day, DAF peroxidase activity in leaf tissue extracts from Col-0, *gpa1-4* and *gpa1-4/agb1-2* was 75% higher than in the control treatment (5 nL L^{-1}) ($P \leq 0.05$), while activities in extracts from *agb1-2* and in all genotypes from the 125 nL L^{-1} treatment were not significantly different from controls (Fig. 3c). After 2 d, DAF peroxidase activity was increased twofold or more in all genotypes treated with 175 nL L^{-1} O_3 ($P \leq 0.01$) (Fig. 3d). Activity in the 125 nL L^{-1} treatment after 2 d was higher only in the Col-0 and *gpa1-4/agb1-2* genotypes compared with the control treatment ($P \leq 0.05$).

The K_m for DAF and V_{max} increased in Col-0 following exposure to 125 nL L^{-1} O_3 for 2 d while smaller responses in the *gpa1-4/agb1-2* line were not statistically significant (Supporting Information Table S4). The K_m for H_2O_2 was similar in the 5 and 125 nL L^{-1} O_3 treatments in both genotypes. V_{max} for H_2O_2 was significantly higher with elevated O_3 in Col-0, but not in *gpa1-4/agb1-2*.

IWF peroxidase responses to O_3

After exposure to 175 nL $L^{-1}O_3$ for 1 or 2 d, APX activity in IWF samples from all genotypes was two- to threefold greater than controls ($P \leq 0.001$) (Fig. 4a,b). Treatment with 125 nL L^{-1} O_3 for 1 d resulted in two- and threefold higher APX activities in *gpa1-4* and *gpa1-4/agb1-2* plants compared with controls ($P \leq 0.05$) (Fig. 4a). Similar results were observed after the second day of treatment with 125 nL L^{-1} while APX activity in IWF extracts increased more than fourfold in *agb1-2* plants ($P \leq 0.001$) (Fig. 4b).

There were no significant differences in DAF activity in IWF extracts among O_3 treatments after a single 7 h exposure ($P \geq 0.05$) (Fig. 4c). After the second day of O_3 exposure, activity was elevated in IWF extracts from Col-0 plants in the 175 nL L^{-1} treatment, but not among the other genotypes or O_3 treatments (Fig. 4d).

DISCUSSION

Even though transcription of the G-protein α- and β-subunit genes, as well as *RGS1*, was stimulated by O_3, most gene expression, biochemical and physiological responses to O_3 were similar among Col-0, *gpa1-4, agb1-2* and *gpa1-4/agb1-2* plants in this study. This included effects on visible injury, leaf tissue ion leakage, A_n and g_s. Booker *et al.* (2004) also found that visible injury and suppression of biomass production due to chronic O_3 was similar among G-protein mutant lines and Col-0, although epinasty of fully expanded leaves, a common response to O_3 in *Arabidopsis* (Sharma & Davis 1994), was less in *gpa1-4* plants. Transcription of the α and β G-protein subunits was clearly stimulated by 125 nL L^{-1} O_3 (Table 2), suggesting a role for these proteins in oxidative stress responses to low O_3 concentrations. Tosti *et al.* (2006) found that transcription of *AGB1* increased 1.8-fold in Col-0 plants after treatment with 300 nL L^{-1} O_3 for 3 h, while *GPA1* was stimulated 3.2-fold at 12 h after the O_3 treatment. In another study, mRNA levels of *AGB1* and *GPA1* rapidly increased in Col-0 treated with 350 nL L^{-1} O_3 for 6 h, although the increase in *AGB1* was transitory (Joo *et al.* 2005). However, there was little indication in our study that down-regulation of photosynthetic genes or up-regulation of oxidative stress genes in response to 125 nL L^{-1} O_3 required signalling through G-protein α- and β-subunits, in contrast with interpretations in previous studies with high O_3 (Mahalingam & Fedoroff

2003; Joo *et al.* 2005). Even without an influence on O_3-induced changes in transcription, a role for G-proteins in oxidative stress signalling is not excluded. Subtle but significant increases in intercellular APX activities in the 125 nL L^{-1} treatment in the null mutants suggested that α and β proteins have a role in regulating APX activity at low O_3 concentrations (Fig. 4).

In our study, g_s was not significantly different among genotypes even though stomatal density was 40% lower in *gpa1-4* plants compared with Col-0 (Table 4). Nilson & Assmann (2010) found that g_s in *gpa1-4* was 20% lower than in Col-0, but this was for plants measured at a PPFD of 1000 μmol m^{-2} s^{-1}, a higher light level than in our study. Lower g_s would decrease O_3 flux and possibly injury, but this was not observed in our *gpa1-4* plants at the PPFD level used (*ca.* 500 μmol m^{-2} s^{-1}). Stomatal opening was reported to be larger in *gpa1-4* compared with Col-0 (Wang *et al.* 2001), which could have compensated for the lower stomatal density in *gpa1-4* and explain the similarity in g_s between genotypes. Vahisalu *et al.* (2010) found that an O_3-triggered rapid transient decrease in g_s that requires SLAC1 and the protein kinase OST1 was unchanged in *gpa1-4* and *agb1-2* plants, indicating that O_3-induced activation of anion fluxes was not dependent on these G-protein genes. There does not appear to be a tight linkage between O_3 effects on stomatal closure and these G-proteins.

Some different responses to O_3 between G-protein mutant and Col-0 plants have been reported previously (Booker *et al.* 2004; Joo *et al.* 2005). Joo *et al.* (2005) found that *gpa1* mutants were less injured by 500 or 700 nL L^{-1} O_3 for 3 h compared with Col-0 and exhibited lower leaf tissue ion leakage and ROS production in response to 350 nL L^{-1} O_3 for 6 h. In contrast, *agb1-2* mutants were more sensitive to O_3 than Col-0 in these treatments. We found that relative ion leakage from leaves increased similarly among genotypes within O_3 treatment regimes following exposure to 175 or 300 nL L^{-1} O_3, although *agb1-2* and *gpa1-4/agb1-2* lines had slightly higher values (Fig. 1). Measurements of physiological and biochemical changes at lower O_3 concentrations that do not cause extensive tissue damage may be more informative and relevant to typical O_3 air pollution.

Col-0 and G-protein mutant plants all showed similar inhibition of A_n and g_s following exposure to 125 and 175 nL L^{-1} O_3, without exhibiting severe tissue damage or ion leakage, particularly in the 125 nL L^{-1} treatment (Figs 1 & 2). Our measurements of A_n and g_s were generally comparable with previous studies. For example, A_n in control plants was similar to values observed in Nilson & Assmann (2010), but higher than values reported in Booker *et al.* (2004) and Brosche *et al.* (2010). The g_s value in control plants was similar to those reported in other studies (Booker *et al.* 2004; Brosche *et al.* 2010; Nilson & Assmann 2010), although it was about half that observed by Overmyer *et al.* (2008).

A decrease in g_s with high O_3 exposures has been attributed in part to direct effects on guard cell regulation of stomatal opening possibly via influences of H_2O_2 and indirectly abscisic acid (ABA) (Overmyer *et al.* 2008; Vahisalu *et al.* 2010). In addition, O_3 effects on stomatal regulation may arise from changes in photosynthesis (Kangasjarvi *et al.* 2005). Ozone is known to inhibit Rubisco activity and photosynthetic electron transport, which has negative feedback effects on g_s via increases in C_i (Pell, Schlagnhaufer & Arteca 1997; Fiscus *et al.*

2005). In our experiment, C_i decreased slightly with O_3, suggesting that decreased A_n under elevated O_3 could be attributed in small part to stomatal limitation. This effect is post-transcriptional as there were no significant changes in gene expression for Rubisco or related subunits, although light-harvesting complex, putative oxygen-evolving proteins 1 and 2, nonphotochemical quenching and chlorophyll synthesis genes were down-regulated by 125 nL L^{-1} O_3 after 2 d (Table 3). Our results for Rubisco expression differed from previous studies that employed high O_3 exposures (Conklin & Last 1995; Mahalingam *et al.* 2006; Tosti *et al.* 2006), although Ludwikow *et al.* (2004) found no change in Rubisco expression with high O_3 treatment. Our results were similar to findings by Mahalingam *et al.* (2005) for photosynthetic light reaction component responses to O_3. Decreased chloroplast gene expression was a rapid response to O_3 stress (Mahalingam *et al.* 2005), which may be indicative of a general down-regulation of photosynthetic processes in response to oxidative stress that is perceived in the chloroplast due to changes in redox state in the apoplast (Kangasjarvi *et al.* 2005). In this case, it may have been accompanied by an inhibition of carboxylation capacity from suppressed Rubisco activity (Pell *et al.* 1997; Fiscus *et al.* 2005; Feng *et al.* 2008).

The 125 nL $L^{-1}O_3$ treatment induced a number of changes in gene expression associated with oxidative stress and ROS signalling (Table 3, Supporting Information Table S1). These responses included up-regulation of peroxidases, thioredoxins, glutathione S-transferases, NAD(P)H oxidases and ascorbate oxidase. Similar responses have been observed in previous gene expression studies conducted at high O_3 concentrations with *Arabidopsis* (Tamaoki *et al.* 2003; Ludwikow *et al.* 2004; Mahalingam *et al.* 2005; Tosti *et al.* 2006). While the G-protein α subunit may be required for activation of membrane-bound NAD(P)H oxidases and the subsequent production of ROS (Joo *et al.* 2005), transcription of the genes for these proteins was stimulated by O_3 in both Col-0 and *gpa1-4/agb1-2* plants.

Many of the genes up-regulated by O_3 appear to be associated with ET, JA and SA signalling (Kangasjarvi *et al.* 2005). Acting as second or third messengers of O_3-induced gene expression, ET, JA and SA antagonistically interact to propagate or counter injury, and these relationships can change depending on the level of oxidative stress, the specific ROS encountered and concentration of the phytohormones (Rao & Davis 2001; Kangasjarvi *et al.* 2005; Mullineaux & Baker 2010). Expression of ET, JA and SA synthesis genes was stimulated (Supporting Information Table S2), including some genes induced by ET (*ATGSTF3*), SA (peroxidase 33, *NPR1*) and ET/JA, but suppressed by SA (*GRX480*, *ATGSTF7*) (Tamaoki *et al.* 2003). *NPR1* is a key regulatory component that functions at the intersection of multiple defence pathways involving SA, JA, redox regulation and possibly the *WRKY70* transcription factor (Dong 2004) that we found up-regulated by O_3 after 3 h. Clearly, a complex network and interplay of gene signalling is involved in plant O_3 responses, but there was no indication that G-protein *gpa1/agb1* null mutants responded differently from Col-0 in these analyses, thus suggesting independent response pathways.

Several genes encoding proteins involved in controlling JA and SA biosynthesis, as well as markers for various ROS, PCD and defence responses, were up-regulated by O_3 (Supporting Information Tables S1 & S2). Notably, up-regulation of *EDS1* and *ATNUDT7* occurred in response to O_3, as has been observed previously (Jambunathan & Mahalingam 2006; Tosti *et*

al. 2006). Induction of these genes, both of which are sensitive to ROS production, appears to encode proteins that have antagonistic control of SA biosynthesis and subsequent changes in oxidative stress outcomes affecting cell death processes (Mullineaux & Baker 2010). Similarly, changes in the levels of MAP kinases influence plant sensitivity to O_3 by affecting expression upstream of antioxidant genes, ET and SA (Kangasjarvi *et al*. 2005). Exposure to 125 nL L^{-1} O_3 for 2 d increased expression of *FMO1*, which has been proposed to be a marker for PCD and other cell defences against oxidative stress (Olszak *et al*. 2006). In contrast to Olszak *et al*. (2006), we found that transcription of *FMO1* was dramatically increased by O_3 after 2 d, which was consistent with Tosti *et al*. (2006). Several other markers of PCD were induced by O_3 in our study (Supporting Information Table S1).

In previous studies, O_3 rapidly stimulated activity of a cationic APX in IWF extracts from *Sedum album* leaves, which was accompanied by a decrease in AA and increase in DHA concentrations in IWF samples (Castillo & Greppin 1988). A two-step model for extracellular peroxidase regulation in response to O_3 was suggested by Castillo & Greppin (1988). It was proposed that extracellular APX (cationic isozyme) activity increased rapidly in response to O_3 followed later by increased syringaldazine peroxidase (anionic isozyme) activity. Differential isozyme expression is thought to result from enzyme activation initially due to changes in Ca^{+2} regulation followed by increases in peroxidase gene expression and protein synthesis (Castillo, Penel & Greppin 1984; Heath 2008). However, in *Arabidopsis*, peroxidase activity regulation in response to O_3 differed from the two-step model. In our experiment, there was a rapid and sustained increase in APX activity in IWF extracts with O_3, but not DAF peroxidase activity, whereas in leaf tissue extracts, both DAF peroxidase and APX activities increased, with DAF activity increasing with exposure duration. Similarly, in *Arabidopsis* treated with 100 nL L^{-1} O_3, APX activity in leaf tissues rapidly increased, followed by an increase in guaiacol peroxidase activity 1 day later (Kubo *et al*. 1995). Activity of APX saturated at 150 nL L^{-1} O_3 compared with higher O_3 concentrations, but guaiacol peroxidase activity continued to increase with exposure duration. It was suggested that APX was needed for early protection, while guaiacol peroxidase was needed as damage expanded (Kubo *et al*. 1995).

Increased APX activity with O_3 may not require transcriptional regulation. Gene expression of *APX1* with O_3 increased in some studies (Kubo *et al*. 1995; Tamaoki *et al*. 2003; Ludwikow *et al*. 2004; Mahalingam *et al*. 2005), but not in others (D'Haese *et al*. 2006;Tosti *et al*. 2006). The lack of change and even down-regulation of APX gene expression in our study were noteworthy (Table 3). D'Haese *et al*. (2006) found a similar lack of effects on APX gene expression in *Arabidopsis* following exposure to 150 nL L^{-1} O_3 for 2 d, as did Tosti *et al*. (2006) following exposure to 300 nL L^{-1} O_3 for 6 h. In our study, APX gene expression was not up-regulated by O_3, but enzyme activity rapidly and substantially increased, especially in IWF extracts, suggesting post-transcriptional regulation mechanisms were involved (Mittler & Zilinskas 1994; Yoshimura *et al*. 2000). Plant responses to O_3 involving APX activity changes are likely important for countering O_3 toxicity. Transgenic tobacco plants expressing antisense RNA for cytosolic APX were more sensitive to acute O_3 (Orvar & Ellis 1997). The adaptive significance of changes in DAF peroxidase activity remains to be clarified.

In conclusion, it was found that *gpa1-4*, *agb1-2* and *gpa1-4/agb1-2* null mutations had only minor influences on plant sensitivity to O_3, as indicated by gene expression, visible injury, relative ion leakage, A_n, g_s and peroxidase activity. Alteration in stomatal density in *gpa1-4* and *agb1-2* mutants did not have a discernible effect on g_s or O_3 sensitivity. Both APX and DAF peroxidase activities in leaf tissue extracts increased with elevated O_3, but the response of DAF peroxidase increased with O_3 exposure duration. APX activity in IWF extracts increased with elevated O_3, but not DAF peroxidase.

It remains unresolved how O_3 toxicity combines with ROS-induced genetic control of cell death to result in plant damage (Kangasjarvi *et al*. 2005). There is evidence from studies with the *Arabidopsis flu* mutant, which generates $^1O_2$ in the chloroplast following a dark/light shift, that stress symptoms were not attributable to cell damage caused by $^1O_2$, but rather resulted from an indirect role of this ROS as a stress signal that might non-enzymatically generate peroxy derivatives that serve as stable second messengers or by inducing the production of fatty acid metabolites through activation of oxidative enzymes (op den Camp *et al*. 2003). Second messengers arising from O_3 reactions could trigger multiple stress responses (Rao & Davis 2001). Signalling cascades activated by H_2O_2 release have been identified in plants (op den Camp *et al*. 2003). Oxidative bursts mediated by G-proteins following acute O_3 exposure may play a role in propagating such ROS signals in the cell, especially to the chloroplast, but a number of ROS signalling pathways are not dependent on this mechanism (Joo *et al*. 2005). It was evident from our study that numerous transcriptional changes related to oxidative stress, ROS signalling and phytohormone responses occurred in plants lacking α/β G-proteins as well as inhibition of vital physiological processes such as A_n. The rapid and sustained increase in APX activity in IWF extracts suggested that a disturbance of the redox state in this location had occurred, which was also transduced to leaf tissues, as indicated by changes in DAF peroxidase activity. G-proteins may modulate these responses but their overall influence on O_3 toxicity in plants appears to be minimal under the environmental conditions utilized in the study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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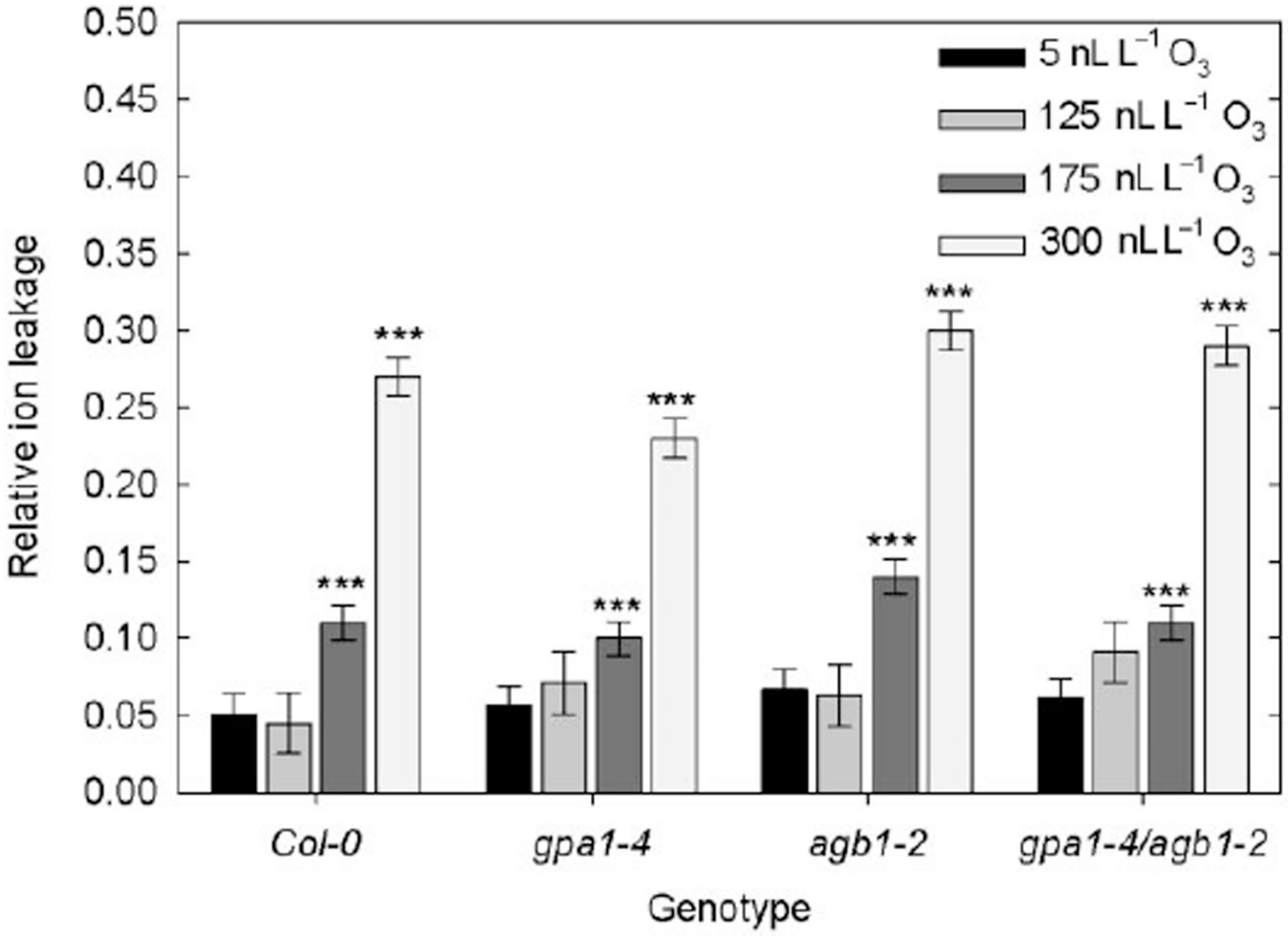

Figure 1.

Relative ion leakage from leaves of Col-0, *gpa1-4, agb1-2* and *gpa1-4/agb1-2* plants following a 7 h exposure to 5, 125, 175 or 300 nL L^{-1} O_3. Statistically significant pairwise comparisons with the respective control treatment (5 nL L^{-1}) are indicated as: ***, $P \leq 0.001$. Values are LSMEANS ± SE.

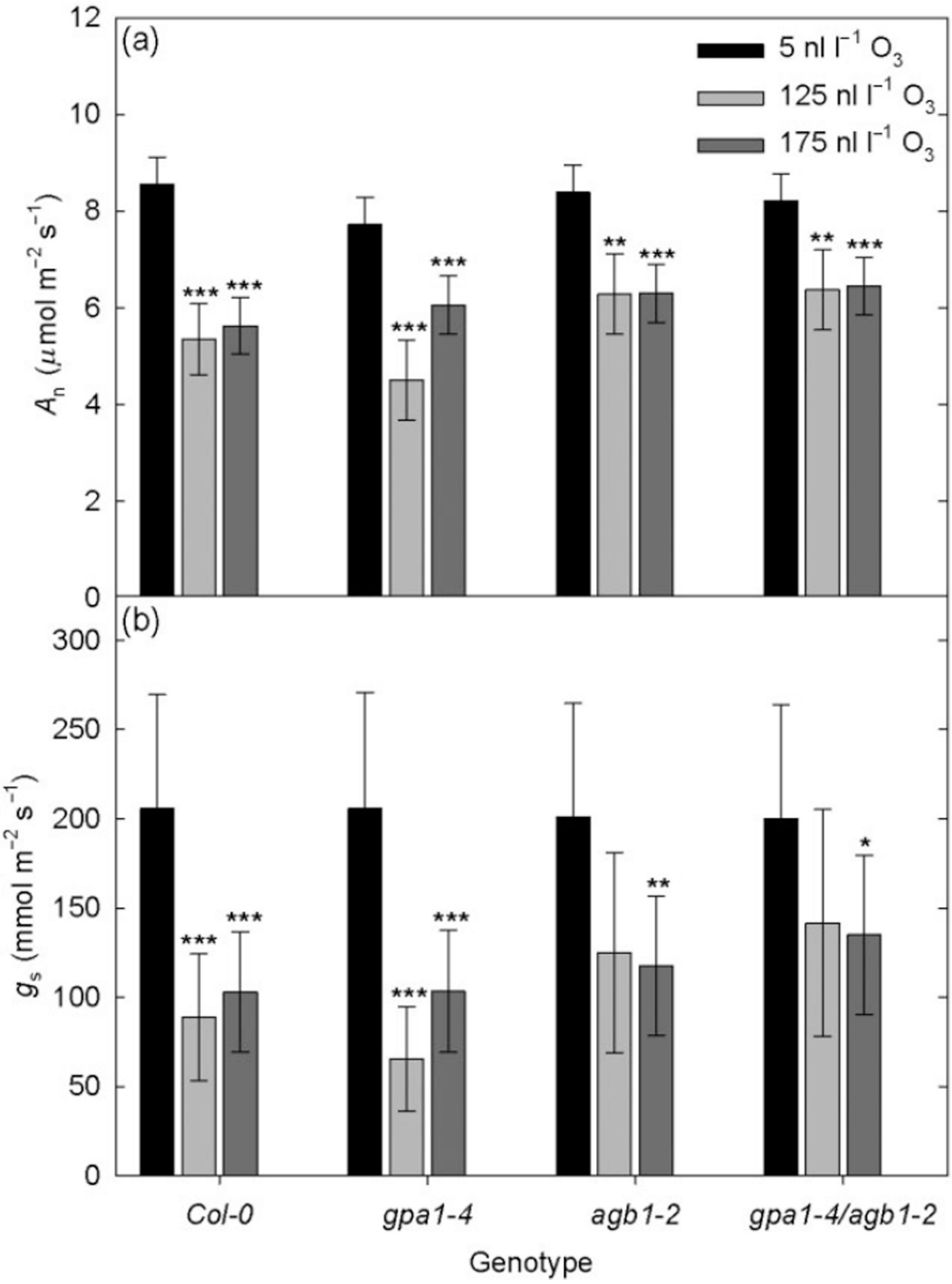

Figure 2.

Net photosynthesis (A_n) (a) and stomatal conductance (g_s) (b) of Col-0, *gpa1-4, agb1-2* and *gpa1-4/agb1-2* following treatment with 5, 125 or 175 nL L^{-1} O_3 for 7 h d^{-1}. Average results after 1 and 2 d of exposures are shown. Statistically significant pairwise comparisons with the respective control treatment (5 nL L^{-1}) are indicated as: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. Values are LSMEANS ± SE.

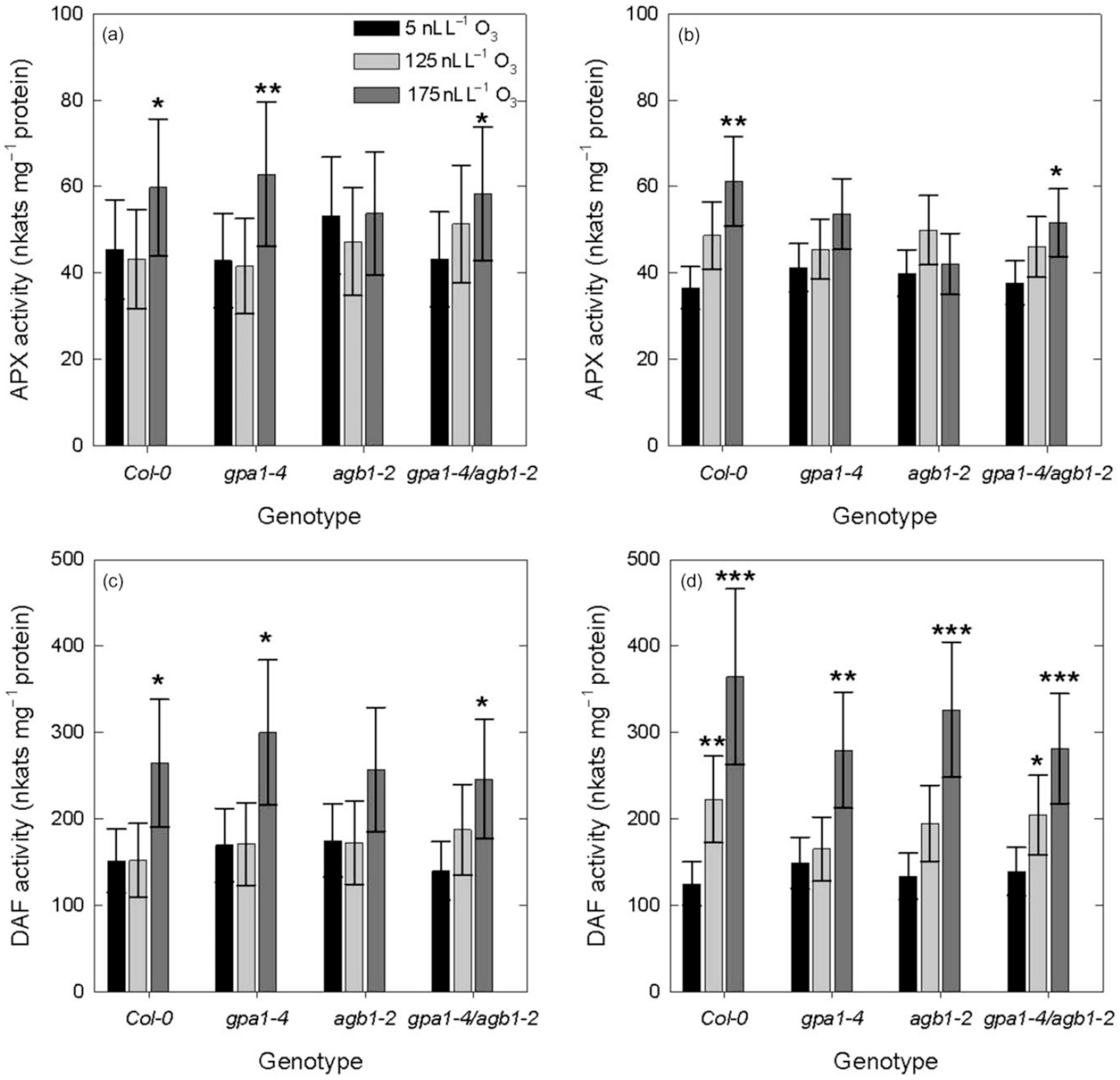

Figure 3.

Ascorbate (a,b) and DAF (c,d) peroxidase activity in extracts of leaf tissues from Col-0, *gpa1-4, agb1-2* and *gpa1-4/agb1*-2 plants treated for one (a,c) and two (b,d) 7 h periods with either 5 (control), 125 or 175 nL L^{-1} O_3. Values are LSMEANS ± SE and the statistically significant pairwise comparisons with the respective 5 nL L^{-1} O_3 control treatment are indicated as: *, $P \leq 0.05$; **, $P \leq 0.001$; ***, $P \leq 0.0001$. APX, ascorbate peroxidase; DAF, 2,7-diaminofluorene.

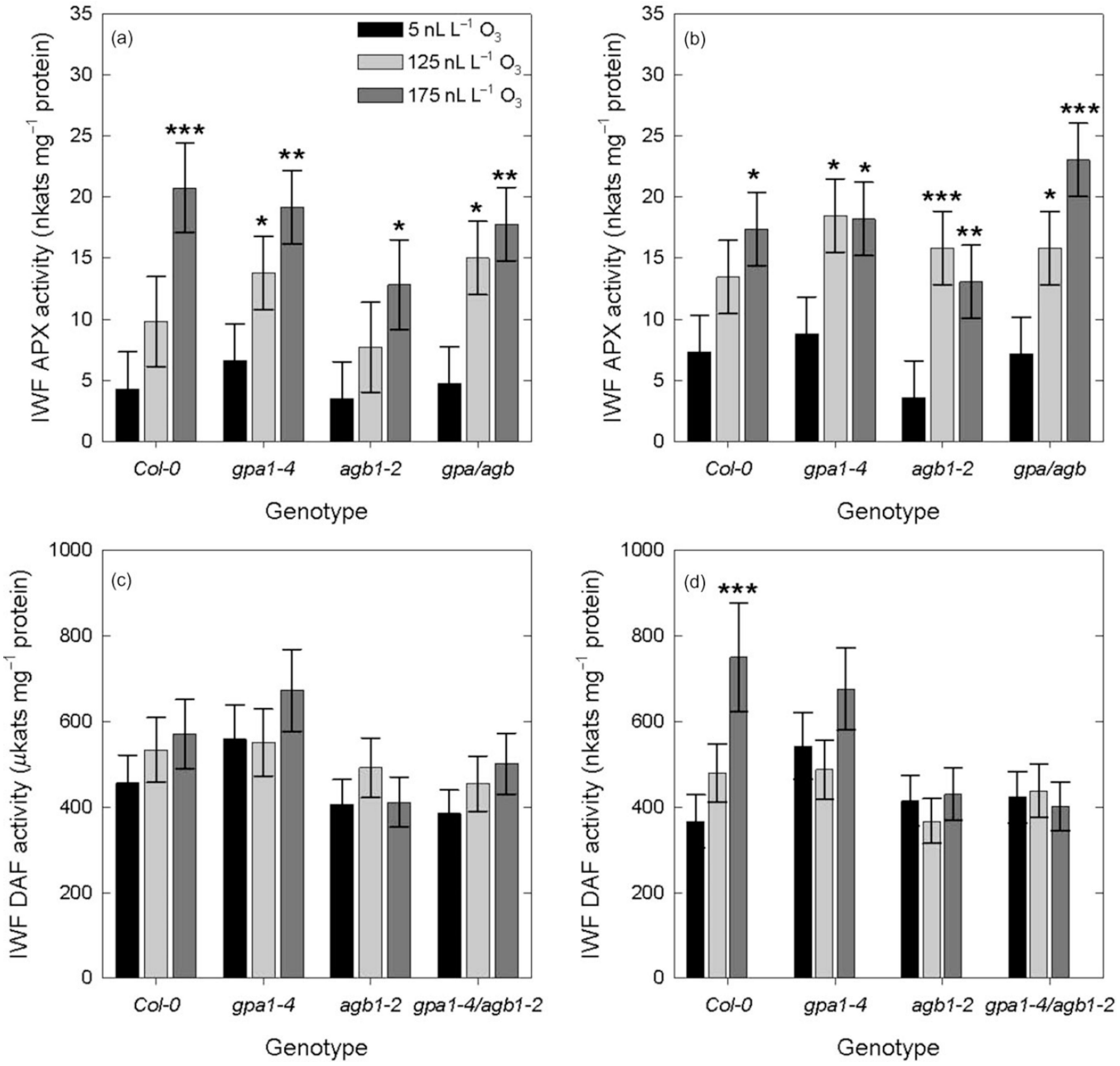

Figure 4.

Ascorbate (a,b) and DAF (c,d) peroxidase activity in IWF extracts from Col-0, *gpa1-4, agb1-2* and *gpa1-4/agb1*-2 plants treated for one (a,c) and two (b,d) 7 h periods with either 5 (control), 125 or 175 nL L^{-1} O_3. Values are LSMEANS ± SE and the statistically significant pairwise comparisons with the respective control (5 nL L^{-1} O_3) are indicated as: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. APX, ascorbate peroxidase; DAF, 2,7-diaminofluorene; IWF, intercellular washing fluid.

Table 1

List of genes up- or down-regulated in *gpa1-4/agb1-2* versus Col-0 at 3 h and 2 d after O_3 treatments began (genotype effect, $P \leq 0.05$)

AGB ID	Gene symbol	Name or description	Fold change 3 h	Fold change 2 d
G-protein				
At2g26300	*GPA1*	G-protein α-subunit 1	−7.2	−13.0
At4g34460	*AGB1*	GTP-binding protein β 1	−6.7	−4.7
Stress response				
At1g20440	*COR47*	Responds to cold, water stress and ABA	1.7	ns
At5g22500	*FAR1*	Fatty acid reductase 1; responds to salt, wounding; suberin biosynthesis	1.7	ns
At2g38870		Predicted pathogenesis-related protein-6; responds to fungus, wounding	ns	−2.9
Transporter				
At5g17700		Transmembrane transport	−1.5	ns
Metabolism				
At3g05170	*AtPGM*	Phosphoglycerate/bisphosphoglycerate mutase family protein	ns	3.1
Unknown				
At3g16660			−1.5	ns
At3g16670			−2.5	ns
At3g22060		Responds to ABA	ns	−2.1

ABA, abscisic acid; ns, not statistically significant.

Table 2

Effect of 125 nL L^{-1} O_3 for 3 h and 2 d on gene expression in the G-protein complex ($P \leq 0.05$)

AGB ID	Gene symbol	Genotype	Fold change 3 h	Fold change 2 d
At2g26300	*GPA1*	Col-0	1.5[a]	ns
At4g34460	*AGB1*	Col-0	2.2	ns
At3g26090	*RGS1*	Col-0	1.6	ns
		gpa1-4/agb1-2	1.6	ns

[a]Fold-change marginally significant ($P \leq 0.09$).

O_3 × genotype interactions were not statistically significant.

ns, not statistically significant.

Table 3

List of peroxidase and ascorbate oxidase genes up- or down-regulated at 3 h and 2 d after exposure of Col-0 and *gpa1-4/agb1-2* plants to 125 nL L^{-1} O_3 (two-way ANOVA, $P \leq 0.05$)

AGB ID	Gene symbol	Name	Fold change 3 h	Fold change 2 d
At4g09010	*APX4*	Ascorbate peroxidase	ns	−1.5
At4g32320	*APX6*	Ascorbate peroxidase	ns	−1.4
At1g77490	*tAPX*	Ascorbate peroxidase	ns	−2.4
At1g71695		Peroxidase 12	ns	−2.9
At5g40150		Peroxidase 26	−1.8	ns
At3g49110/20		Peroxidase 33/34	ns	1.9
At5g19880		Peroxidase 42	ns	1.5
At1g14550		Peroxidase 46	1.8	ns
At4g37520/30		Peroxidase 50	ns	2.6
At1g65980	*TPX1*	Thioredoxin-dependent peroxidase 1	ns	1.7
At1g60740/ At1g65970	*TPX2*	Thioredoxin-dependent peroxidase 2	ns	1.7
At2g31570	*ATGPX2*	Glutathione peroxidase 2	ns	1.6
At2g43350	*ATGPX3*	Glutathione peroxidase 3	ns	1.6
At3g63080	*ATGPX5*	Glutathione peroxidase 5	1.5	ns
At4g11600	*ATGPX6*	Glutathione peroxidase 6	ns	1.7
At4g39830		Ascorbate oxidase	3.7	2.3

O_3 × genotype interactions were not statistically significant ($P \geq 0.05$). Fold-change values are averages of Col-0 and *gpa1-4/agb1-2* responses.

ns, not statistically significant.

Table 4

Stomatal densities in abaxial leaf surfaces of Col-0 and G-protein mutant lines (*gpa1-4, agb1-2* and *gpa1-4/agb1-2*)

Genotype	Stomatal density (stomata mm^{-2})
Col-0	191 ± 17
gpa1-4	108 ± 17**
agb1-2	244 ± 17*
gpa1-4/agb1-2	175 ± 17

Values are the LSMEANS ± SE.

Significant differences between G-protein mutant lines and Col-0 are indicated as: *, $P \leq 0.05$; **, $P \leq 0.01$.